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Spent yeast as an efficient medium supplement for fucoxanthin and eicosapentaenoic acid (EPA) production by *Phaeodactylum tricornutum*

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Abstract

Fucoxanthin and eicosapentaenoic acid (EPA) are high-value compounds that can be found in the marine diatom *Phaeodactylum tricornutum*. Yet, the growth rate of this microalga is relatively low under photoautotrophic conditions. The purpose of this study was to evaluate the feasibility of using spent yeast, the second-major by-product of the brewing industry, as a useful substrate for *P. tricornutum* cultivation. Different pretreatments, concentrations of spent yeast, and initial cell densities of *P. tricornutum* were investigated. After 12 days of cultivation in f/2 medium, *P. tricornutum* supplemented with 1.33 g L^{-1} preautoclaved spent yeast yielded 3.28 times more fucoxanthin (5.97 mg L^{-1}) and 3.55 times more EPA (16.82 mg L^{-1}) than *P. tricornutum* grown without the yeast (fucoxanthin 1.82 mg L^{-1}, EPA 4.64 mg L^{-1}). Nutrient analysis showed that the nitrogen and phosphorus released by the spent yeast were consumed over time. Overall, spent yeast effectively promoted the fucoxanthin and EPA yields of *P. tricornutum*.

Keywords Spent yeast · *Phaeodactylum tricornutum* · Fucoxanthin · EPA · Nutrients analysis

Introduction

A group of simple but widely distributed life forms, algae are a promising source of novel, biologically active substances and essential compounds for human nutrition (Cardozo et al. 2007). Fucoxanthin, a xanthophyll commonly found in brown seaweeds and diatoms, has strong antioxidant activity and has been successfully used in the pharmaceutical, nutraceutical, and cosmeceutical fields (Galasso et al. 2017). Eicosapentaenoic acid (EPA, C20:5) is a well-accepted polyunsaturated fatty acid with proven health benefits, yet supplies are insufficient to satisfy nutritional daily requirements (Tocher et al. 2019). The marine diatom *Phaeodactylum tricornutum* contains both of these highly valued compounds (Zhang et al. 2018).

Phaeodactylum tricornutum has served as a model organism in many studies. Gene transfer methodology has been established (Apt et al. 1996) and evolved (Karas et al. 2015) over the years. The molecular tools required have been invented (Zaslavskaia et al. 2000) and optimized (Slattery et al. 2018). Whole-genome sequencing, assembly and annotation of *P. tricornutum* have been performed by Bowler et al. (2008), resulting in the successful application of gene modification strategies like CRISPR/Cas 9 (Stukenberg et al. 2018) and TALENs (Weyman et al. 2015). These studies provide new approaches of genetically engineering this specific alga. Cryopreservation (Koh et al. 2015) makes it possible to maintain mutant libraries for genetic stability preservation at low cost. All these studies indicate that production of this species for commercial applications may soon be possible.

For the purpose of increasing biomass productivity, many studies have focused on *P. tricornutum*'s ability to thrive under different culture conditions. The strategies tested include utilizing a variety of carbon or nitrogen sources (Yongmanitchai and Ward 1991; Fábregas et al. 1997; Ceron-Garcia et al. 2005; Nur et al. 2019), adopting different cultivation systems indoors or outdoors (Zamalloa et al. 2012; Silva Benavides et al. 2013), applying genetic modification (Seo et al. 2018),

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and using different culture methods like batch culture, fed-batch, and continuous fermentation (Carvalho and Malcata 2005; Cerón-García et al. 2013). Researchers have attempted to use inexpensive substrates like agro-industrial waste for *P. tricornutum* cultivation (Fábregas et al. 1997; Veronesiv et al. 2017; Nur et al. 2019; Patel et al. 2019). Fábregas et al. (1997) used the soluble fractions of wheat, rye, and boiled potato flours to culture *P. tricornutum* semi-continuously; algal productivity (4.1×10^9 cells L^{-1} day^{-1}) was 2.4 times higher in the potato group than in the autotrophic group. Veronesiv et al. (2017) utilized agro-zootechnical ultrafiltered digestate for *P. tricornutum* cultivation; the final biomass was similar to those obtained in f/2 with 10 times nitrogen enrichment. When palm oil mill effluent was used as a substrate for *P. tricornutum* growth, after optimization by the Box–Behnken response surface methodology and the addition of urea, the cell density reached 8.13×10^6 cells mL^{-1} (Nur et al. 2019). Patel et al. (2019) added birch and spruce hydrolysates to promote *P. tricornutum* growth. The highest EPA production (256 mg L^{-1}) and productivity (19.69 mg L^{-1} day^{-1}) were observed with spruce hydrolysates, which were 3 and 10 times higher, respectively, than those obtained from cultivation without glucose and from photoautotrophic cultivation.

Saccharomyces yeast, also known as spent yeast, is the second-major by-product in the beer production process. The total amount of spent yeast biomass produced in lager fermentation is about 1.7–3.0 kg m^{-3} of beer (Fillaudeau et al. 2006; Ferreira et al. 2010). China has been the leading country in beer production for over a decade; in 2018, the production volume was above 380 million kiloliters. With such tremendous volumes of beer being produced, large amounts of spent yeast will continue to be generated. Spent yeast is in high demand as a protein source, so the sludge is usually collected and later sold. The general applications for spent yeast have been summarized by Ferreira et al. (2010) and Rakowska et al. (2017); they include fish nutrition, food ingredients, and a substrate for microorganisms. Spent yeast contains high amounts of protein, nucleic acids (mostly RNA), B complex vitamins, and minerals, making it suitable as a useful alternative nitrogen source for chemical production (Ferreira et al. 2010; Chen et al. 2011). To the best of our knowledge, no studies have been conducted on using spent yeast for photoautotrophic algae cultivation.

Nitrogen is required for the synthesis of proteins, nucleic acids, chlorophyll, and other cellular components. Nitrogen limitation would cause declines in cellular pigments, photochemical efficiency in the PS II center, and the synthesis of a subunit of RuBisCO (Geider et al. 1993). In addition to some inorganic forms of nitrogen, microalgae can also take up micromolecular organics like urea, amino acids, and purines (Yongmanitchai and Ward 1991; Perezgarcia et al. 2011). Phosphorus is an important component of cellular membranes and nucleic acids. It also participates in energy metabolism by regulating enzymes such as RuBisCO (Anwaruzzaman et al. 1995; Singh and Satyanarayana 2011). A phosphorus deficiency would compromise the Calvin cycle, leading to low biomass productivity. The Redfield ratio (nitrogen:phosphorus) of f/2 medium is above 16:1 (Guillard 1975), so algae would run out of phosphorus after several days of cultivation. Like other eukaryotic phytoplankton under phosphorus limitation, *P. tricornutum* produces alkaline phosphatase to break down organic phosphorus (Dyhrman and Ruttenberg 2006; Lin et al. 2013). Moreover, spent yeast may harbor growth factors capable of stimulating the growth and division of *P. tricornutum*. Adenosine and 2′-deoxyadenosine, both of which are present in yeast extracts, are isolated from humus and have been identified as efficient growth promotors that can boost the growth of *P. tricornutum* even under low concentration (Komoda et al. 1983).

The aim of this study is to examine the possibility of obtaining higher yields of fucoxanthin and EPA per batch by using spent yeast as a substrate for *P. tricornutum* culture. We applied different pretreatments of spent yeast to evaluate whether handling technique could affect fucoxanthin and EPA production. Then, we determined the optimal concentration of spent yeast needed to achieve the highest production of the compounds of interest.

Materials and methods

Microalgal strain and culture conditions

The alga *Phaeodactylum tricornutum* (strain no. FACHB-863), purchased from the Freshwater Algae Culture Collection at the Institute of Hydrobiology, China, was grown in f/2 medium (Guillard 1975) under 60 μmol photons m^{-2} s^{-1} at 20 ± 1 °C. The f/2 peptone-methylamine test medium (pH 7.4) contained: 1 g L^{-1} peptone, 1 g L^{-1} methylamine, 10 g L^{-1} agar, and 1 L filtered natural seawater. The malt test medium (pH 7.4) contained 5 g L^{-1} peptone, 10 g L^{-1} malt extract, 10 g L^{-1} agar, 750 mL filtered natural seawater, and 250 mL double-distilled water. About 200 μL of *P. tricornutum* was spread on the surface of the f/2 peptone-methylamine test medium and on the malt test medium to test for the presence of marine bacteria and fungi, respectively (Andersen et al. 1997). Each test plate was incubated at 28 °C for 7 days to account for slow-growing microorganisms. Axenic culture was acquired by using a combination of ampicillin (100 μg mL^{-1}) and kanamycin (100 μg mL^{-1}) for 3 days. The photoperiod was set at 12 h of light and 12 h of dark. Natural seawater with a salinity of 28‰ was obtained from Chuanshi Island, Fujian Province, China, filtered through 0.45-μm PTFE membranes to remove fine particles

and then autoclaved. After cooling to room temperature, presterilized f/2 nutrients were added.

All chemicals used in this study were from readily available commercial sources and of analytical grade.

Spent yeast and pretreatments

Spent yeast was separated from the culture medium following fermentation and then washed several times using double-distilled water to remove the soluble fractions. The cells were freeze-dried for 2 days and stored in reagent bottles at 4 °C until use.

To make the spent yeast usable for alga and to prevent other microorganisms from growing, we performed five pretreatments (Table 1).

Experimental design

For each experiment, *P. tricornutum* precultured in a 2-L conical flask to the middle of the logarithmic phase was used as inoculum. Sterilized natural seawater (400 mL) enriched with f/2 nutrients was inoculated with 50 mL of the inoculum in a 500-mL conical flask. Light, photoperiod, and temperature conditions were as stated previously. The flasks were manually shaken 6 to 8 times daily during the light period. Cultures were maintained for 12 days, and samples were taken every 3 days for biomass, fucoxanthin, and fatty acid composition analysis. Supernatants were subjected to further nutrient analysis.

Experiments were conducted, in triplicate, as follows.

First, to evaluate the effectiveness of the different pretreatments, *P. tricornutum* was cultured in f/2+ SBA, f/2+SBAr, f/2+AST, f/2+SHBA, f/2+HAST, and f/2, and the spent yeast concentration was set at 0.66 g L^{-1}.

Next, to determine the initial concentration of yeast required for optimal growth, f/2 was enriched with 0, 0.22, 0.66, 1.33, 2.22, and 4.44 g L^{-1} of the SBA-pretreated spent yeast.

Finally, to assess the different initial cells at different concentrations to shorten culture time, f/2 was enriched with 0, 1.33, and 2.22 g L^{-1} of the SBA-pretreated spent yeast started at 5×10^5 and 1×10^6 cells mL^{-1}, respectively.

To evaluate the effects of spent yeast on *P. tricornutum* cultivation, samples of non-inoculated f/2 medium containing 1.33 g L^{-1} SBA-treated spent yeast ("Treated−"; see Table 4) and 1.33 g L^{-1} SHBA-treated spent yeast ("SHBA−"; see Table 4) were taken every 3 days from day 0 to day 12. The filtrate was saved for nutrient analyses.

Analytical methods

Biomass

Twenty-five milliliters of culture was harvested by centrifugation at 10,000×*g* for 5 min. The sample was then washed twice with double-distilled water to remove any salt. After freezing at − 80 °C for 2 h, the pellets were lyophilized for 2 days.

Fucoxanthin detection

Ten milliliters of culture at each tested condition was filtered onto 50 mm diameter glass-fiber filters (1.2 μm pore size, Shanghai Xingya Membrane Enterprise, Shanghai, China). Filters were immediately frozen at − 80 °C and then lyophilized for 2 days. To minimize fucoxanthin degradation, each freeze-dried sample was extracted under dim light in a 10-mL tube with 4.00 mL methanol and several 3-mm-diameter steel beads. Tissuelyser JXFSTPRP-64 (Shanghai Jingxin Industrial Development Co., Ltd., Shanghai, China) was applied to ensure a complete extraction. Extracts were passed through syringe filters (nylon membrane, 0.22 μm pore size) prior to analysis with ultra-performance liquid chromatography (UPLC). The analytical procedure was performed as described by Avula et al. (2015), using a Waters Acquity UPLC system (Waters Corp., USA) that included a binary solvent manager, a sample manager, a column compartment, and a PDA detector.

FAME analysis

After biomass evaluation, lipids were extracted from samples of each tested condition. The fatty acid methyl esters (FAME) conversion protocol and analytical methods were performed as described in Gupta et al. (2013), with the following modifications.

An Agilent (Agilent Technologies, USA) 7890B gas chromatograph system with a flame ionization detector (FID) was used in the FAME analysis. The GC was equipped with a capillary column (SP2560 100 m × 0.25 mm × 0.2 μm). Nitrogen was used (instead of helium) as the carrier gas at a flow rate of 1.2 mL min^{-1} (constant flow). The injector was

Table 1 Different pretreatments of spent yeast applied in this study

Pretreatment	Description
SBA	Dried cells sterilized, then added to sterilized seawater
SBAr	Dried cells sterilized, added to sterilized seawater, then removed
AST	Dried cells added to filtered seawater, then both sterilized
SHBA	Dried cells sterilized, high-pressure homogenized, then added to sterilized seawater
HAST	Dried cells high-pressure homogenized, added to filtered seawater, then both sterilized

maintained at 250 °C, and a sample volume of 1 μL was injected with a 10:1 split ratio. The oven was programmed to heat up from 140 °C (5 min hold) to 250 °C (12.5 min hold) at a rate of 4 °C min^{-1}.

Nutrient consumption

After the cells were collected, the supernatants were stored at − 20 °C until further use. A continuous flow analyzer (San++; Skalar, The Netherlands) was used to measure total nitrogen (TN), nitrate (NO_3^-) and nitrite (NO_2^-), total phosphorus (TP), and phosphate (PO_4^{3-}).

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 20 (IBM Corp., USA).

Data were considered significantly different at $P < 0.05$ (one-way ANOVA, followed by a Duncan's test), and each reported value represents the mean ± standard deviation of three biological replicates.

Results

Effect of spent yeast pretreatment on fucoxanthin and EPA production in *P. tricornutum*

The chemical parameters of the f/2 medium containing different pretreated spent yeast are shown in Table 2.

High-pressure homogenization can lead to complete cell lysis, which was confirmed by microscope observations. As a result, the SHBA method released more chemicals than any of the other methods. Autoclaving changed the chemical composition slightly, mostly in TN and TP. As for the pretreatments without homogenization, intact yeast cells could be observed under the microscope. At the start of this experiment, only partial components, according to TN and TP, had been released.

All of the pretreatments except the SHBA method boosted the fucoxanthin and EPA contents (Fig. 1). The volumetric contents of fucoxanthin and EPA, as well as the percentage of EPA, increased over the time.

The SBA pretreatment was the most efficient. By day 12, the fucoxanthin (4.58 mg L^{-1}) and EPA (11.78 mg L^{-1}) contents per batch of *P. tricornutum* were 2.65 and 3.01 times greater, respectively, than the amounts from cultures grown in conventional f/2 medium. Comparing data obtained from SBA and SBAr, the yeast contents were gradually released during cultivation, causing the differences in the final contents of fucoxanthin and EPA in those two groups.

The second-best way to pretreat the spent yeast was the AST method. Fucoxanthin (3.71 mg L^{-1}) and EPA (9.84 mg L^{-1}) concentrations in the AST pretreated culture were 2.14- and 2.52-fold higher, respectively, than those found in the cultures grown in f/2 culture medium (Fig. 1).

In the SHBA pretreatment, the spent yeast was contaminated by microorganisms introduced during the high-pressure homogenization process. Thus, this pretreatment was omitted from the experiments, and no samples were taken except on day 0. Meanwhile, more chemicals were released and autoclaving eliminated microorganisms in the HAST method, but *P. tricornutum* exhibited poor growth. Although the EPA content was slightly higher than that of the algae cultured in the conventional medium, the fucoxanthin content was similar.

Thus, the pretreatment of spent yeast is important for *P. tricornutum* cultivation. As the highest concentrations of fucoxanthin and EPA were obtained using the SBA method, this pretreatment was adopted for the following experiments.

Effect of spent yeast concentration on fucoxanthin and EPA production in *P. tricornutum*

To evaluate the effects of SBA-pretreated spent yeast concentration on algal growth, the microalga was cultivated in f/2 medium with different concentrations (0 to 4.44 g L^{-1}) of spent yeast (Fig. 2).

Table 2 Characteristics of f/2 culture medium enriched with different pretreated spent yeast, before and after cultivation. All experiments were performed in triplicate ($n = 3$). SD standard deviation, − no data were collected

Culture conditions	TN (mean ± SD) (mg L^{-1})		NO_3^- and NO_2^- (mean ± SD) (mg L^{-1})		TP (mean ± SD) (mg L^{-1})		PO_4^{3-} (mean ± SD) (mg L^{-1})	
	Initial	End	Initial	End	Initial	End	Initial	End
f/2+SBA	22.29 ± 0.53	12.47 ± 0.56	12.24 ± 0.98	0.27 ± 0.11	2.46 ± 0.12	1.93 ± 0.06	1.23 ± 0.08	0.12 ± 0.07
f/2+SBAr	21.63 ± 1.21	7.05 ± 0.32	12.08 ± 0.76	1.97 ± 0.56	2.32 ± 0.28	0.74 ± 0.14	1.19 ± 0.13	0.09 ± 0.05
f/2+AST	28.68 ± 1.42	16.12 ± 0.76	12.18 ± 0.38	2.86 ± 0.22	3.42 ± 0.36	2.13 ± 0.07	1.35 ± 0.22	0.11 ± 0.02
f/2+SHBA	39.54 ± 1.17	–	12.41 ± 0.24	–	4.23 ± 0.28	–	1.68 ± 0.16	–
f/2+HAST	34.12 ± 1.86	19.32 ± 1.52	11.88 ± 1.06	4.26 ± 0.71	3.85 ± 0.30	2.24 ± 0.21	1.43 ± 0.11	0.05 ± 0.02
f/2	12.84 ± 0.98	1.95 ± 0.82	11.97±0.78	1.85 ± 0.53	1.19 ± 0.11	0.21 ± 0.06	0.99 ± 0.08	0.08 ± 0.01

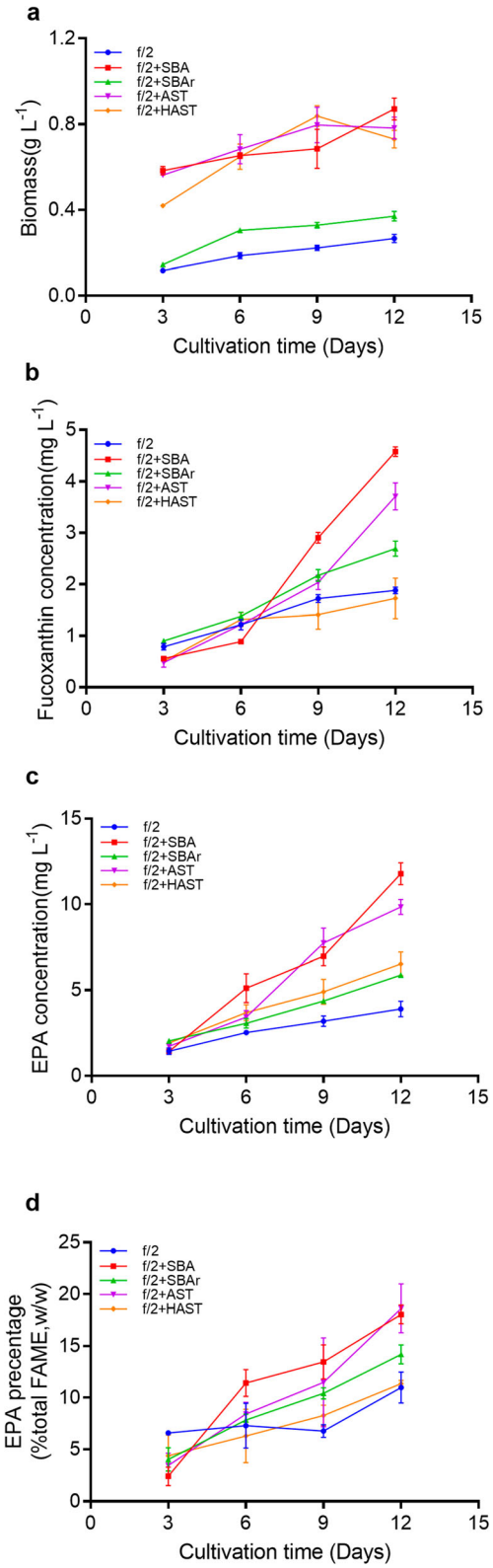

Fig. 1 Effects of the different spent yeast pretreatments on *P. tricornutum* **a** biomass, **b** fucoxanthin production, **c** EPA production, and **d** EPA content (% of total FAME). All experiments were performed in triplicate ($n = 3$). Error bars indicate standard deviation

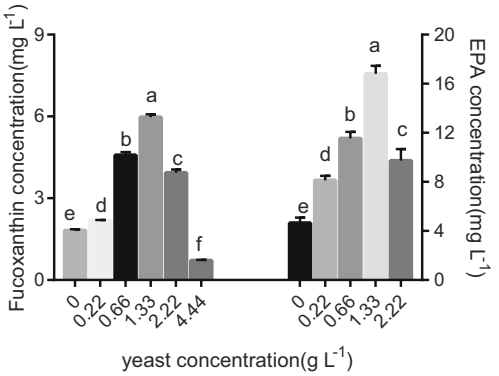

Fig. 2 Effects of spent yeast (SBA-pretreated) concentrations on fucoxanthin and EPA production after 12 days of culture. All experiments were performed in triplicate ($n = 3$). Error bars indicate standard deviation. Different letters indicate significant differences ($P < 0.05$, Duncan's test)

Fucoxanthin content increased monotonically from 1.82 to 5.97 mg L^{-1} as the spent yeast concentration increased from 0 to 1.33 g L^{-1}, but it decreased as the yeast concentration rose further. Likewise, EPA content increased from 4.74 to 16.82 mg L^{-1} as the spent yeast concentration increased from 0 to 1.33 g L^{-1}, and then decreased when it reached 2.22 g L^{-1}. Moreover, no EPA was detected after 3 days in the 4.44 g L^{-1}groups.

Effect of initial cell density on fucoxanthin and EPA production in yeast-adding *P. tricornutum*

These experiments were conducted to determine whether high initial cell densities of *P. tricornutum* can shorten cultivation time and increase tolerance of higher spent yeast concentrations. Exponential-phase *P. tricornutum* grown under photoautotrophic conditions had a cell density of approximately 6×10^6 cells mL^{-1}. Initial cell densities were thus set at 5×10^5 and 1×10^6 cells mL^{-1}—the "low" and "high" cell densities, respectively—and SBA-pretreated spent yeast concentrations were 0, 1.33, and 2.22 g L^{-1}.

Final volumetric productivities and concentrations for both fucoxanthin and EPA under different culture conditions are compared and summarized in Table 3.

Increases in final fucoxanthin and EPA production were expected with increased inoculum size, but this was not the case. The fucoxanthin and EPA parameters showed similarity; when the yeast concentration was 1.33 g L^{-1}, the results for the low-cell-density groups were significantly higher ($P < 0.05$) than those of the high-cell-density groups. With the higher yeast concentration (2.22 g L^{-1}), the results for the high-density groups were significantly higher than those of the low-density groups. No significant differences were observed between the low- and high-density groups in the yeast-free system.

Table 3 Growth parameters of *P. tricornutum* with different initial cell densities and yeast concentrations after 12 days of culture. All experiments were performed in triplicate ($n = 3$). *SD* standard deviation. Different letters indicate significant differences ($P < 0.05$, Duncan's test)

Parameters		0 g yeast per liter		1.33 g yeast per liter		2.22 g yeast per liter	
		L	*H*	*L*	*H*	*L*	*H*
Fucoxanthin (mean ± SD)	$\overline{P}$	0.15 ± 0.01e	0.15 ± 0.01e	0.53 ± 0.01a	0.46 ± 0.01b	0.32 ± 0.01d	0.42 ± 0.02c
	X	1.93 ± 0.06e	2.11 ± 0.04e	6.46 ± 0.03a	5.86 ± 0.12b	3.93 ± 0.01d	5.34 ± 0.27c
EPA (mean ± SD)	$\overline{P}$	0.35 ± 0.02d	0.33 ± 0.01d	1.14 ± 0.09a	1.09 ± 0.10b	0.78 ± 0.06c	0.89 ± 0.07c
	X	4.52 ± 0.23c	4.72 ± 0.09c	14.44 ± 1.10a	13.49 ± 1.08a	9.72 ± 0.07b	11.43 ± 0.81b

$\overline{P}$ productivity during cultivation, mg L^{-1} day^{-1}, *X* production concentration at the end of cultivation, mg L^{-1}; *L* initial cell density at 5×10^5 cells mL^{-1}, *H* initial cell density at 1×10^6 cells mL^{-1}

The most compatible yeast concentration was 1.33 g L^{-1} (Table 4). The fucoxanthin *X* (6.46 mg L^{-1}) and $\overline{P}$ (0.53 mg L^{-1} day^{-1}) resulted in 3.35- and 3.51-fold higher values, respectively, than those obtained with the f/2 culture. The EPA *X* (14.44 mg L^{-1}) and $\overline{P}$ (1.14 mg L^{-1} day^{-1}) at this concentration were also the highest—approximately 3.19- and 3.30-fold higher compared with the f/2 medium. With 2.22 g L^{-1} spent yeast, the final fucoxanthin and EPA production in the high-density treatment were 2.76- and 2.53-fold higher, respectively, than those in the low-density group. Increasing initial cell density indeed resulted in improved tolerance to high yeast concentration.

Productivity is a suitable evaluation parameter for production as it takes the cultivation time into account. As seen in Fig. 3, fucoxanthin and EPA productivity exhibited a similar pattern, indicating that the spent yeast functioned mainly as a substrate to promote algal cell growth. The fucoxanthin productivity of *P. tricornutum* was 0.15 mg L^{-1} day^{-1} in the f/2 medium without spent yeast, which was higher than the results reported by Wu et al. (2016) (0.12 mg L^{-1} day^{-1}) and Nur et al. (2019) (0.04 mg L^{-1} day^{-1}). While the productivities in the groups without spent yeast tended to decline over time, those in the 1.33 g L^{-1} yeast-added groups increased. This is likely because of insufficient nutrients in the yeast-free groups, and reduced light absorption in the 2.22 g L^{-1} groups.

Roles of spent yeast in *P. tricornutum* cultivation

The final fucoxanthin and EPA contents produced in the f/2 medium with 1.33 g L^{-1} of spent yeast were 3.28 and 3.55 times higher, respectively, than those produced in the f/2 medium without yeast (Fig. 2). Our results suggest that the presence of spent yeast was important for the accumulation of these highly valued compounds.

To understand the effects of spent yeast on the cultivation of *P. tricornutum*, nutrients were analyzed (Tables 2 and 4).

In both tables, nutrient analyses of f/2 medium containing 0.66 g L^{-1} and 1.33 g L^{-1} SHBA-treated spent yeast revealed that spent yeast contained mostly organic forms of nitrogen and phosphorus.

The presence of spent yeast delayed the consumption of NO_3^- (Table 4). Ou et al. (2018) reported that *Aureococcus anophagefferens* had a high absolute uptake rate for NH_4^+ and low half-saturation constants for NO_3^- and urea. *Aureococcus anophagefferens* grew faster on the organic substrate (urea) than on the inorganic substrates (NO_3^- and NH_4^+), indicating that the different types of nitrogen available would tend to have preference for algae. Wheeler et al. (1974) reported that *P. tricornutum* could utilize amino acids as organic substrates (Ala, Glu, Gly, Ser, and Orn). TP can still be removed from the system after the phosphate has been metabolized, which shows that *P. tricornutum* can take up organic phosphorus under a phosphate deficiency.

To evaluate the consumption of nitrogen and phosphorus from the spent yeast, we estimated the contents of nitrogen and phosphorus using non-inoculated f/2 medium containing 1.33 g L^{-1} of SBA-treated spent yeast (Treated+). At 12 days, TN, NO_3^- and NO_2^-, TP, and PO_4^{3-} consumption reached their highest values (32.17, 11.94, 3.57, and 1.29 mg L^{-1}, respectively), using the non-inoculated groups (Treated−) as references. These results suggest that *P. tricornutum* can effectively utilize nitrogen and phosphorus released by spent yeast, and that both elements act as macronutrients in algal cultivation.

The observed fatty acid composition of the total lipids is given in Table 5. All the FAMEs were characterized by the presence of C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, and C20:5. For this *P. tricornutum* strain cultured in f/2 medium, the most abundant fatty acid was C16:0 (25.82%) followed by C18:0 (14.72%); three unsaturated fatty acids—C16:1, C18:1, and C20:5—were present in roughly the same proportions (11–12%).

The presence of spent yeast also altered the composition of fatty acids. The primary fatty acids in the 1.33 g L^{-1} culture were C16:0 (25.82%), C16:1 (14.92%), and C18:0 (14.32%).

Table 4 Comparison of nutrient levels throughout the cultivation period. All experiments were performed in triplicate ($n = 3$). Each value represents the mean ± standard deviation. Different letters indicate significant differences ($P < 0.05$, Duncan's test)

Days	TN (mg L^{-1})			NO_3^- and NO_2^- (mg L^{-1})			TP (mg L^{-1})			PO_4^{3-} (mg L^{-1})		
	Control	Treated+	Treated−	Control	Treated+	Treated−	Control	Treated+	Treated−	Control	Treated+	Treated−
0	12.21 ± 0.44b	30.08 ± 1.22a	30.79 ± 1.04a	11.89 ± 0.23a	12.28 ± 0.99a	12.03 ± 0.33a	1.22 ± 0.25b	3.76 ± 0.15a	3.86 ± 0.23a	1.12 ± 0.07a	1.26 ± 0.14a	1.28 ± 0.19a
3	8.35 ± 0.43c	46.48 ± 0.84b	52.42 ± 1.63a	6.51 ± 0.34c	8.71 ± 0.52b	12.53 ± 0.21a	0.06 ± 0.01c	4.20 ± 0.18b	5.12 ± 0.24a	0.04 ± 0.01b	0.16 ± 0.02b	1.34 ± 0.15a
6	5.18 ± 0.28c	40.73 ± 0.82b	59.75 ± 0.76a	4.48 ± 0.39c	6.35 ± 0.32b	12.45 ± 0.73a	0.05 ± 0.01c	3.65 ± 0.17b	5.88 ± 0.76a	0.03 ± 0.02b	0.05 ± 0.01b	1.31 ± 0.32a
9	4.53 ± 0.11c	35.31 ± 1.59b	61.16 ± 3.67a	3.49 ± 0.52b	2.28 ± 0.26c	12.66 ± 0.42a	0.05 ± 0.02c	3.21 ± 0.22b	6.02 ± 0.68a	0.05 ± 0.03b	0.06 ± 0.03b	1.42 ± 0.22a
12	2.42 ± 0.17c	30.54 ± 0.60b	62.71 ± 2.64a	1.95 ± 0.14b	0.69 ± 0.22c	12.63 ± 0.72a	0.03 ± 0.02c	2.69 ± 0.18b	6.12 ± 0.51a	0.02 ± 0.05b	0.04 ± 0.03b	1.29 ± 0.18a
0	SHBA−			SHBA−			SHBA−			SHBA−		
	66.85 ± 2.76			12.96 ± 0.75			6.41 ± 0.38			2.41 ± 0.36		

Control, algae-inoculated in f/2 medium; Treated+, algae-inoculated f/2 medium containing 1.33 g L^{-1} SBA-treated spent yeast; Treated−, non-algae-inoculated f/2 medium containing 1.33 g L^{-1} SBA-treated spent yeast; SHBA−, non-algae-inoculated f/2 medium containing 1.33 g L^{-1} SHBA-treated spent yeast

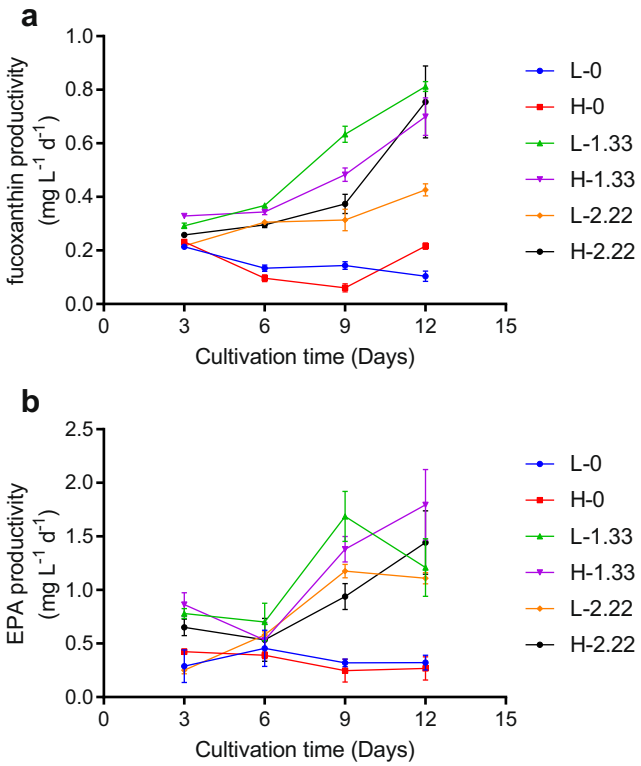

Fig. 3 **a** Fucoxanthin productivity and **b** EPA productivity of *P. tricornutum* under different concentrations of spent yeast with low and high initial cell density. *L* initial cell density at 5×10^5 cells mL^{-1}, *H* initial cell density at 1×10^6 cells mL^{-1}. All experiments were performed in triplicate ($n = 3$). Error bars indicate standard deviation

A significantly higher proportion of C20:5 (16.04%) was present in this treatment than in the conventional culture (10.67%), a 1.5-fold difference.

Discussion

Spent yeast, a by-product from breweries, can serve as a useful substrate for obtaining more fucoxanthin and EPA per batch of cultured *P. tricornutum*. We found that different pretreatments of spent yeast affected the biomass, as well as fucoxanthin and EPA production, of *P. tricornutum.* The AST and HAST pretreatments were less effective than the SBA method. This may be because of the Maillard reaction, a non-enzymatic chemical reaction that occurs through the condensation between a reducing sugar and a nitrogen-containing molecule. The reaction reduces the nutritional value of food and generates a group of antinutritive and toxic compounds called Maillard reaction products (MRPs). When water activity is between 0.6 and 0.7, the MRP generation rate increases exponentially (O'Brien et al. 1989). We speculated that during the autoclaving step in the AST and HAST methods, more water would be available to generate more MRPs in the culture medium. Of several possible mechanisms explaining the MRPs' antimicrobial properties, an effective iron-binding capacity resulting from the MRPs' negative net charge (reviewed in Mu et al. 2016) may be responsible here. Iron is essential for diatom growth because it is needed for metabolic functions (the Calvin cycle and the cytochrome b_6/f complex); an iron deficiency would hinder the microalgae's ability to assimilate nitrogen (Milligan and Harrison 2000; Kustka et al. 2002; Zhao et al. 2018). The selection of chelator is critical because it influences microalgal growth by interfering with iron assimilation (Botebol et al. 2014). The AST and HAST pretreatments adversely affected fucoxanthin and EPA production in *P. tricornutum* compared with the SBA pretreatment (Fig. 1b, c). Thus, a high amount of MRPs in the AST and HAST pretreatments may have negatively affected the growth of *P. tricornutum*, possibly via the MRPs' chelation of iron.

Light is essential for the growth of *P. tricornutum*; Zaslavskaia et al. (2001) and Hayward (1968) demonstrated that *P. tricornutum* cannot survive under chemoheterotrophic conditions. Myers (1953) stated that light would be the final limiting factor for mass culture under constant, controlled conditions. When the average light received by a single cell is less than the saturation light intensity because of the presence of massive cells in the system, the growth rate will eventually decrease (Timm et al. 1991). Nur et al. (2019) reported that the fucoxanthin concentration could be increased by

Table 5 Fatty acid compositional profiles of *P. tricornutum* (% of total FAME, *w/w*) obtained under different spent yeast concentrations at the end of cultivation. *ND* not detected. Data are expressed as means ± standard deviations of three replicates. Different letters indicate significant differences ($P < 0.05$, Duncan's test)

Fatty acids	Spent yeast concentrations (g L^{-1})				
	0	0.22	1.33	2.22	4.44
C14:0	3.91 ± 0.08a	3.86 ± 0.08a	3.45 ± 0.10a	3.27 ± 0.59a	ND
C16:0	35.27 ± 1.09a	27.70 ± 0.13c	25.82 ± 0.20c	32.33 ± 1.16b	35.80 ± 0.34a
C16:1	11.22 ± 0.31b	14.48 ± 0.08a	14.92 ± 0.54a	10.04 ± 1.15b	15.34 ± 0.16a
C18:0	14.72 ± 1.02b	14.46 ± 0.28b	14.32 ± 0.11b	18.25 ± 2.08a	17.81 ± 0.56a
C18:1	11.98 ± 0.75b	6.24 ± 0.07d	6.22 ± 0.23d	7.79 ± 1.11c	20.34 ± 0.22a
C18:2	1.78 ± 0.18bc	2.00 ± 0.03b	1.69 ± 0.05bc	1.90 ± 0.13c	2.63 ± 0.04a
C20:5	11.37 ± 0.25b	10.67 ± 0.08bc	16.04 ± 0.68a	10.08 ± 0.52c	ND
Others	9.75 ± 2.51c	20.59 ± 0.33a	17.54 ± 1.73ab	16.34 ± 0.55b	8.08 ± 1.16c

controlling the light intensity at a relatively low level. McClure et al. (2018) found that at low light intensity, the specific fucoxanthin concentration of *P. tricornutum* (42.8 mg g^{-1}) was greater than at high intensity (9.9 mg g^{-1}). In batch culture of *Monodus subterraneus*, cultivation under low light intensity or high biomass concentration enhanced the proportion of EPA up to 36.7% of fatty acids (Cohen 1994). These indicate that lowering the light intensity could improve fucoxanthin and EPA production. We found that after 12 days of cultivation in the f/2 medium with 1.33 g L^{-1} preautoclaved spent yeast, *P. tricornutum* yielded 3.28 times more fucoxanthin (5.97 mg L^{-1}) and 3.55 times more EPA (16.82 mg L^{-1}) than that produced in the f/2 medium without the yeast (fucoxanthin 1.82 mg L^{-1}, EPA 4.64 mg L^{-1}). We hypothesized that the spent yeast could decrease light penetration, and then increase the fucoxanthin and EPA concentrations at yeast concentrations up to 1.33 g L^{-1} (Fig. 2). When the spent yeast concentration exceeded 1.33 g L^{-1}, the fucoxanthin and EPA concentrations decreased as yeast concentration increased. In addition, though *P. tricornutum* better tolerated high-density yeast when higher initial cell densities were used, the final production of fucoxanthin and EPA was still not comparable to those of the 1.33 g L^{-1} groups (Table 3). It is likely that light became the limiting factor for *P. tricornutum* growth when there were enough nutrients in the system. High concentrations of spent yeast could interfere with light absorption, affecting cell growth and, thus, reducing fucoxanthin and EPA production. Some studies have been reported that carbon form agro-industrial waste, like biomass of birch and spruce, soluble fractions of rye, wheat, potato, and pretreated digestate, could be utilized for *P. tricornutum* growth (Veronesiv et al. 2017; McClure et al. 2018; Patel et al. 2019).

Spent yeast contains a high amount of free amino acids (Tanguler and Erten 2008), and *P. tricornutum* can utilize amino acids (Ala, Glu, Gly, Ser, and Orn) as organic substrates (Wheeler et al. 1974). In the SBAr pretreatment, we removed the spent yeast cells before inoculation; only partial lysate containing amino acids remained in the system. In Table 2, the increase in NO_3^- and NO_2^- was not significant in the spent yeast addition groups when compared with f/2 alone. However, the consumption of TN in the f/2+SBAr pretreatment (approximately 14 mg L^{-1}) was higher than the consumption of NO_3^- and NO_2^- (approximately 10 mg L^{-1}). The difference in consumption values could be because of the consumption of amino acids from partial lysates in the system. Meanwhile, the biomass obtained in the SBAr pretreatment was 0.37 g L^{-1}, which was 1.42 times higher than that obtained in the f/2 medium (Fig. 1a). In addition, the reasons that the proportions of fatty acids changed after the addition of spent yeast might with the amino acid consumption.

Attempts have been made to utilize agro-industrial waste like biomass of birch and spruce and soluble fractions of rye, wheat, potato and pretreated digestate, for *P. tricornutum* cultivation (Fa'bregas et al. 1997; Veronesiv et al. 2017; Patel et al. 2019). But upon further consideration, the biomass of birch and spruce is not massive as that of spent yeast and algal performance with soluble fractions of rye, wheat, potato and pretreated digestate are not comparable with this study. In China, the annual spent yeast produced in the beer industry is over 1.7×10^5 t (dry weight). Almost 600 t of fucoxanthin and 1600 t of EPA could be produced through *P. tricornutum* cultivation, which would fill the gap between the supply and demand.

Conclusions

The present study has shown that spent yeast, the second-major by-product of beer production, can be effectively used for *P. tricornutum* cultivation. Of all the pretreatments and concentrations tested, 1.33 g L^{-1} of preautoclaved spent yeast resulted in the highest yields of fucoxanthin and EPA. The yeast provided *P. tricornutum* with a sustained release of nitrogen and phosphorus and also decreased the light penetration, which helped to boost fucoxanthin and EPA production. The utilization of spent yeast for *P. tricornutum* culture can reduce production costs by increasing productivity, and it provides a new perspective for using *P. tricornutum* to commercially produce valuable compounds like fucoxanthin and EPA.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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